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Extrinsic Labeling Method May Not Accurately Measure Fe Absorption from Cooked Pinto Beans (*Phaseolus vulgaris*): Comparison of Extrinsic and Intrinsic Labeling of Beans

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Isotopic labeling of food has been widely used for the measurement of Fe absorption in determining requirements and evaluating the factors involved in Fe bioavailability. An extrinsic labeling technique will not accurately predict the total Fe absorption from foods unless complete isotopic exchange takes place between an extrinsically added isotope label and the intrinsic Fe of the food. We examined isotopic exchange in the case of both white beans and colored beans (*Phaseolus vulgaris*) with an in vitro digestion model. There are significant differences in $^{58}Fe/^{56}Fe$ ratios between the sample digest supernatant and the pellet of extrinsically labeled pinto bean. The white bean digest shows significantly better equilibration of the extrinsic ^{58}Fe with the intrinsic ^{56}Fe. In contrast to the extrinsically labeled samples, both white and red beans labeled intrinsically with ^{58}Fe demonstrated consistent ratios of $^{58}Fe/^{56}Fe$ in the bean meal, digest, supernatant, and pellet. It is possible that the polyphenolics in the bean seed coat may bind Fe and thus interfere with extrinsic labeling of the bean meals. These observations raise questions on the accuracy of studies that used extrinsic tags to measure Fe absorption from beans. Intrinsic labeling appears necessary to accurately measure Fe bioavailability from beans.

KEYWORDS: Extrinsic labeling; intrinsic labeling; beans; Fe bioavailability; in vitro

INTRODUCTION

Fe deficiency anemia is the most common nutritional disorder worldwide. One of the important contributing factors of Fe deficiency anemia is the low bioavailability of food Fe from daily consumption. The diet may contain less Fe than necessary for metabolic purposes, or even if the diet is rich in Fe, there are often high levels of Fe uptake inhibitors such as phytate and polyphenolic compounds that can exacerbate Fe deficiency by decreasing Fe bioavailability (*1, 2*).

Accurate measurement of Fe absorption is essential to determining requirements and evaluating the factors involved in Fe bioavailability. Isotopic labeling of food has been the method of choice for many human studies. There are two basic choices in isotopic labeling: extrinsic and intrinsic. For plant foods, intrinsic labeling requires hydroponic culture to incorporate an inorganic Fe tracer into the plant; hence, the Fe is present in the natural forms of the plant food (*3*). On the other hand, the extrinsic labeling technique is based on the assumption that complete isotopic exchange takes place between an extrinsically added Fe isotope label and the intrinsic Fe of the food so that the fractional absorption of the extrinsic label by human subjects predicts total Fe absorption (*4*). In some cases, the equilibration of the extrinsic and intrinsic Fe pools may not be achievable for certain foods due to the forms of Fe present. The degree to which Fe can remain soluble and exchangeable during digestion certainly affects the extent of isotopic exchange and thus the final results of Fe uptake.

For a program such as the HarvestPlus (www.harvestplus.org/) program, proper assessment of Fe bioavailability in staple food crops is critical to the success of its biofortification effort. Bean is a major staple crop targeted by HarvestPlus to provide more bioavailable Fe. Donangelo et al. (*5*) compared an extrinsic and intrinsic labeling method for Fe absorption from beans (*Phaseolus vulgaris* L.) by net absorption of the two labels. In their work, extrinsic labels were added to the intrinsically labeled bean meal the day prior to the absorption test, and the labels were allowed to equilibrate overnight. Although in this case,

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the fractional absorption of Fe labels was the same, it is not clear as to the time needed for equilibration or if time was even a factor. As extrinsic labels are frequently added just before the administration of meals, the accuracy of the results in these studies could be in question. The fractional absorption of extrinsic labels may not represent the amount of total Fe absorbed given that incomplete isotopic exchange of the extrinsic label with the internal Fe was possible.

Radioactive isotopes ^{55}Fe and ^{59}Fe were frequently used as extrinsic labels for Fe absorption studies. Because of increasing concerns over radioactivity safety, stable isotopes become preferred labels with the possible accessibility of these isotopes from commercial sources and availability of an inductively coupled plasma mass spectrometry (ICP-MS) system. The addition of a stable isotope with lower natural abundances will allow for more accurate assessment of the isotopic exchange of the extrinsic labels with the internal isotope since the interference from other isobaric ions is low. Thus, ^{58}Fe (natural abundance of 0.282%) was chosen in this study instead of ^{57}Fe (natural abundance of 2.119%). The in vitro digestion Caco-2 cell model had been validated in many cases to predict absorption of Fe in human subjects (*6*). In the present study, we assessed the extrinsic and intrinsic methods for labeling Fe in white and colored beans using a standard in vitro digestion protocol (*7*) to digest bean meals. The isotopic ratio of ^{58}Fe/^{56}Fe was compared between bean digest supernatant and pellet for information on isotopic exchange of the extrinsic label with internal Fe.

MATERIALS AND METHODS

Chemicals, Enzymes, and Hormones. Unless otherwise stated, all chemicals, enzymes, and hormones were purchased from Sigma Chemical Co. (St. Louis, MO). Stable isotope ^{58}Fe in elemental form was purchased from Oak Ridge National Laboratory (Oak Ridge, TN) for extrinsic labeling experiments. For intrinsic labeling of beans, Fe and Zn were supplied as ^{58}Fe(III)-*N*,*N*′-bis(2-hydroxybenzyl) ethylenediamine-*N*,*N*′-diacetic acid (HBED) chelate and ^{70}ZnO (Trace Science International Inc. Richmond, ON Canada). Upon using, the stable isotopes were solubilized using a mixture of sulfuric acid and hydrochloric acid to make a stock solution, and appropriate dilutions were made prior to each experiment.

Extrinsically Labeled Bean Sample Preparation. Pinto (*Phaseolus vulgaris*) and white great northern (*Phaseolus vulgaris*) bean samples were cooked and supplied by Dr. Janet R. Hunt from the U.S. Department of Agriculture—Agricultural Research Service Grand Forks Human Nutrition Research Center (Grand Forks, ND) (*8*). Briefly, pinto and white beans were grown under normal field cultivation conditions in North Dakota or Minnesota (Northarvest Bean Growers Association, Frazee, MN). After they were rinsed with cold water, 454.0 g of dried beans with 1835.0 mL of water, 0.8 g of garlic powder, and 8.0 g of onion powder were cooked in a slow cooker on high heat for 4.5 h. Salt (7.0 g) was added to the meal 30 min prior to boiling. Individual portions containing 100.0 g of cooked beans plus 50 g of cooking liquid were frozen and shipped to the U.S. Department of Agriculture—Agricultural Research Service U.S. Plant, Soil and Nutrition Laboratory (Ithaca, NY) for in vitro digestion experiments.

One day prior to the in vitro digestion experiment, 2.0 g of bean meal (1.33 g of each bean variety plus 0.67 g of the cooking liquid) was weighed into 50 mL tubes, and 3.33 mL of fresh Tropicana orange juice (from a local grocery store with a vitamin C concentration of 1.74 mmol/L) was added to each 2.0 g bean meal when the presence of AA was needed to show a promoting effect on Fe absorption from bean meals. Vitamin C concentration in the same batches of the Tropicana orange juice was determined by high-performance liquid chromatography using a UV−vis detector. The juice for in vitro digestion process was used immediately after opening, and the actual amount of the juice was determined based the calculations of vitamin C concentration so as to have the same Fe to vitamin C molar ratio as

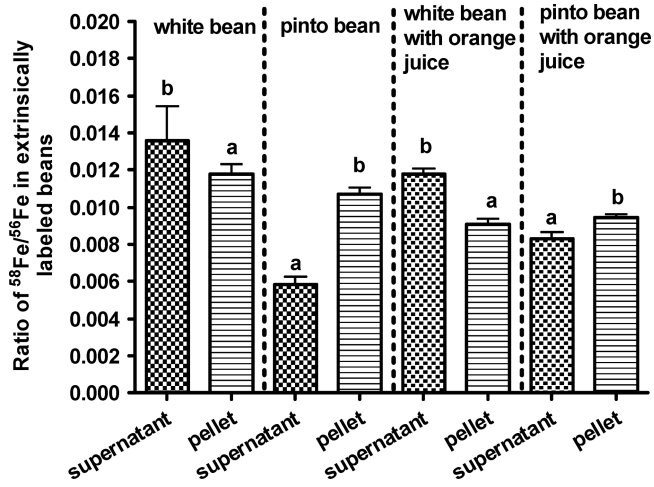

Figure 1. Ratio of ^{58}Fe/^{56}Fe in extrinsically labeled white bean, pinto bean, white bean plus orange juice, and pinto bean plus orange juice meal digest supernatant and pellet. Each pair of columns represents the ratio of ^{58}Fe/^{56}Fe based on the measurement of concentration of ^{58}Fe and ^{56}Fe in both supernatant and pellet of bean meal digest. Each data set represented six separate experiments done on the same day, and values are means ± SEM ($n = 6$). A Graphpad Prism unpaired *t* test was used to compare the data in each pair of columns ($n = 6$, $P < 0.05$).

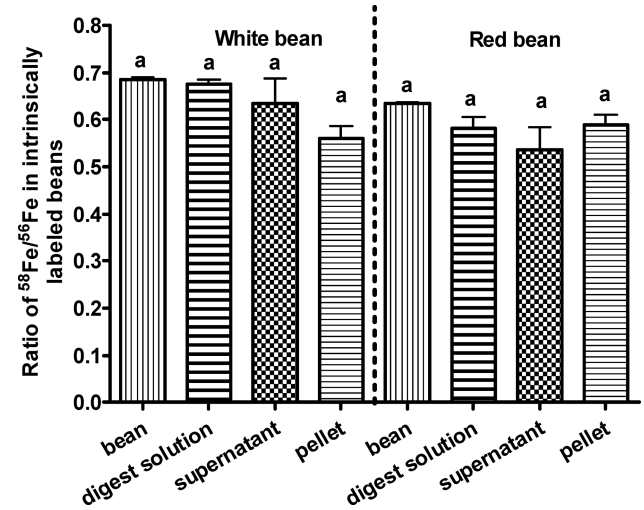

Figure 2. Ratio of ^{58}Fe/^{56}Fe in intrinsically labeled white bean and red bean meal digest. Each column represents the ratio of ^{58}Fe/^{56}Fe based on the measurement of concentration of ^{58}Fe and ^{56}Fe. Each data set represented six separate experiments done on the same day, and values are means ± SEM ($n = 6$). Data were analyzed using the software package GraphPad Prism (GraphPad Software, San Diego, CA). ANOVA with Tukey's post-test was used to compare means among bean powder, bean digest, bean digest supernatant, and pellet ($n = 6$, $P < 0.05$).

that of the human study (*8*). Extrinsic ^{58}Fe stable isotopic labeling was carried out through the addition of 200 μL of 1.0 μg/mL of ^{58}Fe stock solution into each of the samples including white bean, white bean plus orange juice, pinto bean, and pinto bean plus orange juice.

Intrinsically Labeled Bean Sample Preparation. Basically, beans were grown in the similar way as that in the previous human study (*5*). Red bean (*Phaseolus vulgaris* L.) and white bean (*Phaseolus vulgaris* L.) seeds were germinated between layers of water-saturated filter paper in glass-covered Petri dishes at 25 °C in the dark. After hypocotyl and radical emergence, the plantlets were transferred to nutrient solutions of the following composition (in mmol/L): N, 16; K, 6; P, 2; Mg, 1; S, 1; Ca, 4; (in μmol/L) Cl, 50; B, 12.5; Mn, 2; Zn, 2; Cu, 0.5; Mo, 0.1; Ni, 0.1; Fe, 50. Fe in the nutrient solution was

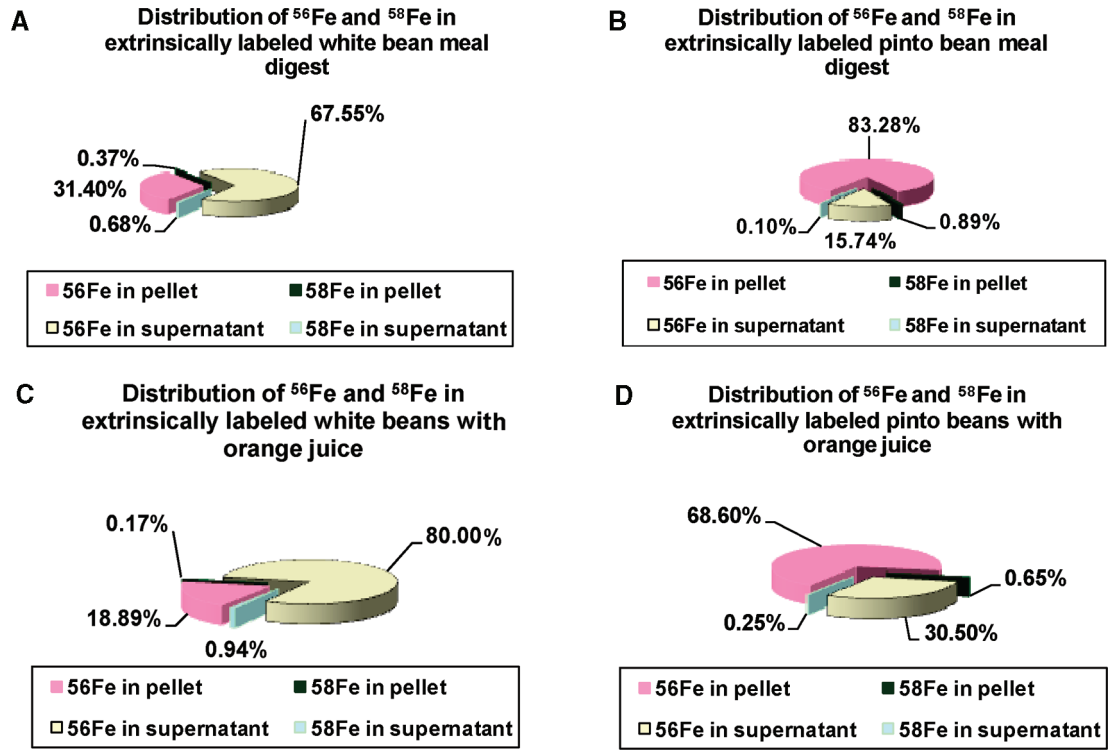

Figure 3. Distribution of relative percentage of ^{56}Fe and ^{58}Fe in extrinsically labeled bean digest. (**A**) Distribution of relative percentage of ^{56}Fe and ^{58}Fe in extrinsically labeled white bean meal digest supernatant and pellet. (**B**) Distribution of relative percentage of ^{56}Fe and ^{58}Fe in extrinsically labeled pinto bean meal digest supernatant and pellet. (**C**) Distribution of relative percentage of ^{56}Fe and ^{58}Fe in extrinsically labeled white bean plus orange juice meal digest supernatant and pellet. (**D**) Distribution of relative percentage of ^{56}Fe and ^{58}Fe in extrinsically labeled pinto bean plus orange juice meal digest supernatant and pellet.

supplied as a regular ferrous sulfate salt until upon flowering, and Zn and Fe were supplied as ^{70}Zn and ^{58}Fe-HBED (10 μmol/L). The reason for using Fe (III)-HBED chelate as a supply of Fe is due to the affinity for Fe, bioavailability, efficacy in relieving Fe overload, superior antimalarial activity, and much lower toxicity of HBED. Seeds were harvested at maturity, and beans were cooked by autoclaving at 100 °C for 15 min. The cooked beans were then lyophilized to dryness and ground to powder for storage in airtight containers at room temperature. One day prior to the in vitro digestion experiment, 1.0 g of each bean variety was weighed into 50 mL tubes.

In Vitro Digestion Method (*7*). To initiate the gastric phase of digestion, 10 mL of salt solutions of 140 mmol/L of NaCl and 5.0 mmol/L of KCl at pH 2.0 was added to each sample. After pH adjustment to 2.0 with 1.0 mol/L of HCl, 0.5 mL of the pepsin solution was added to each tube, and the mixtures were under gastric digestion for 1 h at 37 °C on a rocking platform (model RP-50, Laboratory Instruments, Rockville, MD) located in an incubator, which was circulated with a 5% CO_2, 95% air atmosphere under constant humidity. After 1 h of gastric digestion, the pH of the sample mixture was raised to 5.5−6.0 with 1.0 mol/L of $NaHCO_3$ solution before 2.5 mL of the pancreatin/bile extract solution was added to each mixture. The pH of the mixture was then adjusted to approximately 7.0, and the final volume contained within each tube was adjusted to 15.0 mL by weight using a salt solution of 140 mmol/L of NaCl and 5.0 mmol/L of KCl at pH 6.7. At this point, the mixture was referred to as "digest".

Analysis of Fe Isotopes. Analyses of Fe were conducted using an inductively coupled plasma mass spectrometer (Agilent 7500c ICP-MS, Agilent Technologies, United States) after wet-ashing with HNO_3 and $HClO_4$. To separate sample digest supernatant and pellet, 5.0 mL of the sample digests was transferred into 15 mL centrifuge tubes and centrifuged at 4000*g* for 30 min. The liquid in the top part of the sample, that is, the supernatant of digest, was then transferred into quartz tubes for wet-ashing with HNO_3 and $HClO_4$, dissolution with 2% HNO_3, and analysis by ICP-MS. The pellet of sample digest was collected after removal of all of the supernatant, dried in a drying oven maintained at 105 °C, cooled to room temperature, and weighed into quartz tubes for wet-ashing with HNO_3 and $HClO_4$, dissolution with 2% HNO_3, and analysis by ICP-MS.

Statistical Analyses. Data were analyzed using the software package GraphPad Prism (GraphPad Software, San Diego, CA). Analysis of variance (ANOVA) with Tukey's post-test and nonparametric *t* test was used to compare differences among means within each bean variety. Differences among means were considered significant at $n = 6$ and $P \leq 0.05$.

RESULTS

Extrinsically Labeled Samples. The ratio of ^{58}Fe/^{56}Fe of sample digest supernatant and pellet was different (nonparametric *t* test, $n = 6$, $P \leq 0.05$) within each variety of the extrinsically labeled bean digest both alone and with orange juice present (**Figure 1**). Overall, extrinsically labeled white bean meal digest had less disparity in isotope distribution between supernatant and pellet (**Figure 1**). The difference of ^{58}Fe/^{56}Fe between supernatant and pellet was significant in extrinsically labeled pinto bean meal digest when they were digested alone although ascorbic acid from orange juice helped decrease this difference (**Figure 1**). Incomplete exchange of the extrinsic label ^{58}Fe with the native ^{56}Fe was evidently seen in both white northern bean and pinto bean meal digests but appeared to be greater in the case of pinto bean. Ascorbic acid appeared to moderate the difference in isotope distributions between the supernatant and the pellet of the pinto bean meal digest (**Figure 1**).

With regard to the overall distribution of Fe in extrinsically labeled bean digest, 67.55% of the total Fe in white bean digest was present in supernatant, whereas only 15.74% of total Fe in pinto bean digest went to supernatant (**Figure 3A**,**B**). Upon addition of orange juice, 80.00% of Fe from extrinsically labeled

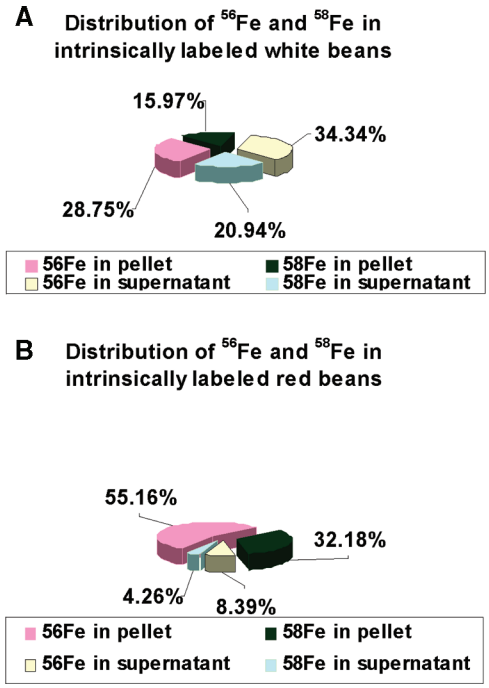

Figure 4. Distribution of relative percentage of ^{56}Fe and ^{58}Fe in intrinsically labeled bean digest. (**A**) Distribution of relative percentage of ^{56}Fe and ^{58}Fe in intrinsically labeled white bean meal digest supernatant and pellet. (**B**) Distribution of relative percentage of ^{56}Fe and ^{58}Fe in intrinsically labeled red bean meal digest supernatant and pellet.

white bean meal digest was in supernatant and 30.50% of Fe from extrinsically labeled pinto bean meal digest was soluble (**Figure 3C**,**D**).

Intrinsically Labeled Samples. The ratio of ^{58}Fe/^{56}Fe in bean, bean meal digest, bean meal digest supernatant, and pellet was statistically the same within each bean variety (ANOVA with Tukey's post-test, $n = 6$, $P \leq 0.05$) in the intrinsically labeled white bean and red bean meal digest (**Figure 2**). White bean and red bean showed similar isotope distribution when they were intrinsically labeled. A 63.09% amount of the total Fe in intrinsically labeled white bean meal digest was in supernatant (**Figure 4A**). Intrinsically labeled red bean meal digest had only 12.65% of Fe in the supernatant (**Figure 4B**). The relative percentage of ^{58}Fe and ^{56}Fe in intrinsically labeled white bean and red bean digest was calculated to be the same (**Figure 4A**,**B**). There was a slightly higher percentage of Fe in the supernatant of the extrinsically labeled bean meal digest as compared to the relevant intrinsically labeled bean digest (**Figures 3A** and **4A** and **3B** and **4B**).

DISCUSSION

Previous studies on validating the extrinsic labeling technique mostly involved the comparison of the net absorption of biosynthetically incorporated Fe isotope with another isotope added extrinsically either during the food preparation stage or offered as a separate drink (*9*). The assumption was that an identical absorption between the biosynthetically labeled (i.e., intrinsic) food Fe and the extrinsically labeled food Fe indicated that a complete isotopic exchange had occurred between the intrinsic and the extrinsic Fe so that the use of an extrinsic tracer will accurately predict Fe absorption from the whole meal. Identical absorption of the extrinsic and intrinsic tags was found in human subjects from foods including maize, wheat, rice, black bean, soybean, and eggs when the extrinsic label was mixed carefully with the studied foods during preparation (*4, 10, 11*). The extent of isotopic exchange was also monitored indirectly by comparing the absorption and appearance of the extrinsic and intrinsic tracers in human plasma. Bjorn-Rasmussen et al. found identical plasma radioactivity of the extrinsic and intrinsic tracers with four human subjects after two of them consumed a wheat bread meal and the other two consumed a soybean meal (*11*). Intrinsic labels from omelets prepared from labeled eggs and white wheat flour were absorbed identically.

The absorption of the extrinsic and intrinsic labels was not always the same though, which shed doubts on the results of studies that used extrinsic labeling technique by assuming a complete isotopic exchange of the extrinsic label with the native Fe. The extrinsic label was 30% more absorbed than the intrinsic label when it was offered in a drink with the intrinsically labeled maize meal. Discrepancies on the absorption of intrinsic and extrinsic labels were also found in soybean meals and maize meals when the extrinsic tag was not added to the intrinsically labeled food during cooking (*4*). In addition, when intrinsically labeled raw rice was extrinsically labeled to make porridge, the extrinsic label was absorbed 40% more than the intrinsic labels (*11*). It seemed that a lot of factors could affect the accuracy of the extrinsic labeling method. It is still not clear as to how long it takes for a complete isotopic exchange to take place for specific meal preparation. The presence of Fe from a contaminated source and Fe absorption inhibitors adds complexity to this matter (*2*).

In the present study, exchange of both biosynthetically or extrinsically labeled Fe (^{58}Fe) with the native ^{56}Fe in bean meal digest was evaluated by comparing isotopic ratios of ^{58}Fe/^{56}Fe during different stages of the in vitro digestion sample preparation stages. Using simple solubility, we compared isotope ratios of ^{58}Fe/^{56}Fe in sample digest supernatant and sample digest pellet. Our hypothesis was that a complete exchange between ^{58}Fe and ^{56}Fe should result in proportionately distributed Fe isotopes in some if not all of the stages of the sample preparation. By the same token, a disparity of the ratios of ^{58}Fe/^{56}Fe between the supernatant and the pellet of the same sample digest indicates a poor exchange or equilibration of ^{58}Fe and ^{56}Fe in the sample preparation stage. For the pinto bean sample, it is clear that equilibration did not complete under the current meal preparation conditions and that significantly better equilibration of the extrinsic isotope occurred in the white bean sample.

It should be ideal for isotopic exchange under pH of 2.0 during the first step of in vitro digestion since Fe is soluble at this pH. Yet, we still observed incomplete exchange of isotopes within our samples. This suggests that it is less likely for complete isotopic exchange to occur in the human stomach where pH can be well above 2.0 and may get to as high as close to neutral during most of the mixing and emptying into the duodenum (*12*). Certainly, it is likely that the formation of micropockets of low pH may occur in the food digesta, but it is unknown if this occurs and if complete solubility and exchange can happen. The accuracy of Fe absorption results from human subjects is thus questionable given that the extrinsic labels were added in a similar fashion (*8*).

The isotopic exchange process has only been studied indirectly by comparing the net absorption of the extrinsic and intrinsic labels (*13*). Can it be assumed that if percent absorption is equal from both methods that equilibration occurred? It seems questionable and especially so when the percent absorption is relatively low, such as less than 5%, which is typical for Fe absorption studies. When extrinsic labels of Fe are added to foods, they may bind to compounds native of the food samples,

such as polyphenolics or phytate. Consequently, Fe will not be freely exchangeable or at the very least vary between the multiple Fe forms. Also, it is possible that the same fractional absorption of extrinsic Fe and native Fe could still be obtained. Hallberg (*2*) showed that exchangeability and bioavailability of Fe are two independent concepts. In their work with rice flour and soil with contaminated Fe, the amount of exchangeable Fe was decreased by more than 10% while the absorption of Fe was increased by 30%, which suggests that extrinsic labeling technique could not be simply validated by comparing absorption. In another study, the addition of different amount of red soil Fe to a meal showed a constant fraction of exchangeable Fe irrespective of the amount of red soil Fe added and total Fe absorbed (*14*). This again suggested that exchangeability and bioavailability of Fe are two independent concepts. It is thus inappropriate to validate the use of extrinsic labeling technique for Fe uptake studies by simply comparing extrinsic and intrinsic label absorption.

In summary, extrinsically added ^{58}Fe did not equilibrate well with the native ^{56}Fe in the pinto bean meal digest and was better equilibrated in the white bean meal digest. Polyphenolic compounds in the seed coat may be responsible for the lack of isotopic equilibration since they could bind Fe to make them unavailable for exchange. Therefore, absorption of extrinsic label under these conditions may not be an accurate measure of bean Fe bioavailability. The intrinsically added ^{58}Fe equilibrated with ^{56}Fe very well in both red bean and white bean digest samples, indicating that this labeling method should be used for single meal bioavailability studies. This type of information is critical for programs such as HarvestPlus, where biofortification of crops with Fe is a primary target, and for many other foods where intrinsic Fe must be administered. Accurate assessment of Fe bioavailability in such projects is crucial to success.

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